Engineered hybrid cardiac patches with multifunctional electronics for the online monitoring and regulation of tissue function

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Device design considerations. Our device was designed to be completely free-standing and provide both recording and stimulation of tissue function, as well as allow spatial release of biochemical factors. The whole fabrication process was accomplished on a nickel sacrificial layer that was removed as the last stage of processing. This yields a completely free-standing, flexible device. To achieve high porosity (>99%) and minimally interfere with tissue formation, pore sizes were ~100 x 1,300 μm^2. SU-8 was chosen both as the substrate and passivation material due to its ease of processing, relative flexibility and transparency which can be later exploited for fluorescent imaging assays. SU-8 passivation allowed electrode exposure only at the terminating pads, which were designed for either recording/stimulating or drug release. Recording/stimulating electrodes were small enough (50x50 μm^2) to enable recording from specific regions within the tissue but not too small so as impedance and thermal noise might interfere with the quality of recording. The entire device included 28 recording/stimulating pads dispersed throughout the long dimension of the device in pairs with distances ranging between 600 and 1500 μm, while 4 larger pads (150x150 μm^2) were designed to accommodate controlled release systems. These large electrodes were placed at equal distances (~4,500 μm) along the device to allow spatially controlled drug release throughout the tissue. Electrode pad size was designed to limit contact to as few cells as possible while avoiding impedance limitations that might occur with smaller electrode sizes. Electrochemical impedance spectroscopy measured in phosphate buffered saline showed an impedance of ~0.1 kΩ at a frequency of 1 kHz (Supplementary Fig S20). Larger electrode pad sizes were designed to allow bulk electroactive polymer deposition for drug release. Two large grounding electrodes were placed on each side of the device to serve as counter electrodes for stimulation purposes. A rough nanoscale layer of titanium nitride (TiN) was deposited on the gold pads to increase their surface area. The electronic network was designed to be flexible to enable

rolling or folding together with the biomaterial scaffold to create a thick 3D structure. Thus, distant electrodes within the electronics are re-organized in space to form a denser electrode network and enable 3D monitoring of tissue function. Overall, the dimensions of the electronic chip were ~20 mm x ~5 mm to enable engineering of thick tissues.

Detailed device fabrication. Free-standing devices were fabricated on the oxide surface of silicon substrates (University wafer, Boston, MA) prior to relief from the substrate. Key steps used in the fabrication process (Figure S1) were as follows: 1) Thin layer metal deposition was performed using a VST e-beam evaporator (VST, Petah Tikva, Israel) to deposit a 20 nm nickel relief layer (Figure S1 b.) 2) A layer of SU-8 photoresist (2 μm, 2002 or 3005, MicroChem Corp., Newton, MA) was deposited over the entire wafer, and photolithography was used to pattern the bottom SU-8 mesh structure (Figure S1c), which was then cured (190 ºC, 35 min). 3) The substrate was then coated with AZ 5214 (MicroChem Corp., Newton, MA) and patterned by photolithography. Cr/Au (5/200 nm) metals were sequentially deposited followed by metal lift-off in NMP to define the different electrodes and connectors of the device (Figure S1d). 5) Subsequently, a second 100 nm thick titanium nitride (TiN) layer was deposited by the same steps used for the gold electrodes, however the TiN thin layer was sputtered using a Penta magnetron sputtering system (Penta Vacuum, Singapore). The substrate was then coated with a uniform layer of SU-8 (5 μm, 3005 or 3025, MicroChem Corp., Newton, MA) and photolithography followed by curing (190 ºC, 35 min) was used to define a passivation layer over the deposited metal electrodes (Figure S1e). 6) The whole device was released from the substrate by etching the nickel layer with nitric acid 21% (w/v) overnight at 25 ºC (Figure S1f). The devices did not adhere to the substrate and floated in the liquid, after which they were transferred to water to wash any remnants of acid and dried in air

before use. Ag/AgCl ink (ALS, Tokyo, Japan) was used for reference electrodes. The device and its components were biocompatible as shown in Supplementary Fig. S21.

SEM. Samples were mounted onto aluminum stubs with conductive paint and sputter-coated with an ultrathin (150Å) layer of gold in a Polaron E 5100 coating apparatus (Quorum technologies, Laughton, UK). The samples were viewed under JSM-840A SEM (JEOL, Tokyo, Japan). Images of higher resolution were obtained using a JSM-6700F SEM (JEOL).

Metallurgical Confocal microscopy. Confocal and metallurgical microscopy images of the electrodes were taken using an Olympus LEXT 4000 confocal microscope (OLYMPUS, Tokyo, Japan).

Atomic force microscopy. Topography measurements were performed by atomic force microscopy (AFM, Molecular imaging Pico Plus, Agilent, Santa Clara, CA) with the use of PicoScan5 software. Scanning was performed on whole devices on wafers.

Electrical resistance measurements. All measurements of conductivity were performed using a Keithley 4200 Semiconductor characterization system (Keithley Instruments, Cleveland, OH) using a two probe set up. Resistance was measured by scanning current as a function of voltage change and extrapolating the resistance. Perturbations were performed and measurements taken before and after and conductivity was compared.

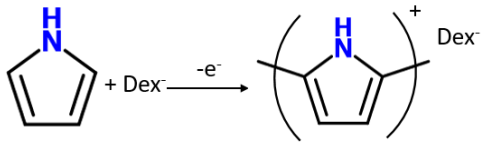
Protein release from chondroitin sulfate. Three hundred mg chondroitin 4-sulfate (CS) (Sigma-Aldrich) were dissolved in 1.14 ml of 1 N NaOH under vigorous stirring. Two hundred and forty μL of the cross-linker EGDGE (Sigma-Aldrich) were then added and mixed thoroughly. The mixture was degassed under vacuum for 10 min, after which it was transferred to a mold. The latter was sealed and placed in an oven at 60°C for 60 min. Cross-linking of the polymer took place and

a gel was formed. After cooling at room temperature, the cylindrical hydrogel was removed from the mold, cut into small slices, washed in double distilled water in glass vials, and left in 4°C for 3 days to obtain equilibrium swelling. The water was replaced daily. Washing was carried out to remove any un-reacted polymer/cross-linker from the gel. All release experiments were conducted at room temperature in PBS, using a custom-made release chamber. The gels were then placed in a solution of lysozyme in double distilled water for 24 hours. One piece of protein loaded gel was removed from the loading medium, blotted dry, rinsed with a PBS buffer, blotted dry once again, and then placed on the device under a binocular. Electrical stimulation (1 V, 10 min on, 20 min off) was applied by creating an electric field between the electrode on to which the gel was placed and one of the ground electrodes. Protein release was followed by taking samples of the medium every 30 min. The samples were replaced by adding an equal volume of buffer to the release chamber. The passive release experiments were conducted in the same way without stimulation. Lysozyme release was quantified by absorbance at 280 nm using a NanoDrop ND-1000 UV-Vis Spectrophotometer (NanoDrop, Wilmington, DE)[S1]. Release was performed using a fully fabricated microECP ($n \geq 3$).

Protein loading analysis. Protein loading was measured by placing cylindrical hydrogels of the same size in a 1 mL protein solution. Both lysozyme and SDF-1 solutions were used with varying concentrations. Protein concentration was measured at the beginning of the experiment and every 24 hours. Protein concentration was assessed by ELISA for SDF-1 and spectrophotometry at 280 nm for lysozyme.

SDF-1 loading and release. SDF-1 was loaded and released in the same manner as lysozyme. SDF-1 was detected using a human CXCL12/SDF-1 ELISA kit (R&D systems, Minneapolis, MN).

Dexamethasone release from Polypyrrole films. The underlying principal Polypyrrole electropolymerization is shown in the following schematic:



PPy/DEX films were grown potentiostatically onto the device electrodes. The electrosynthesis solution consisted of 0.2 M Pyrrole (Alfa Aesar, Ward Hill, MA) and 0.02 M DEX (Sigma-Aldrich, dexamethasone 21 phosphate disodium, used as received) in Milli-Q water of 18 MΩ/cm resistivity. The film was synthesized by applying a constant potential of 1 V for 5 minutes between the device and a counter electrode. The devices containing PPy/DEX films were then rinsed several times with Milli-Q water. The selected PPy/DEX film covered electrode and the ground electrode were connected to a stimulus generator (STG-4002, Multichannel systems), and a voltage of (-0.6) – (-1) V was applied for 10 minutes. A sample was collected 20 minutes after the stimulation was terminated. The amount of released DEX was quantified by absorbance at 242 nm (a characteristic band of DEX) using a NanoDrop ND-1000 UV-Vis Spectrophotometer [S2]. Release was performed using a fully fabricated microECP (n≥3).

Polypyrrole film thickness measurement. PPy/DEX films were grown potentiostatically onto the device electrodes. The electrosynthesis solution consisted of 0.2 M pyrrole (Alfa Aesar, Ward Hill, MA) and 0.02 M DEX (Sigma-Aldrich, dexamethasone 21 phosphate disodium, used as received) in Milli-Q water of 18 MΩ/cm resistivity. The film was synthesized by applying a constant potential of 1 V for varying time lengths. Film thickness was measured using an Olympus LEXT 4000 confocal microscope (OLYMPUS, Tokyo, Japan).

Cell migration assay. Jurkat cells, expressing CXCR4 receptors were serum starved in RPMI medium (RPMI 1640, Biological Industries, Kibbutz Beit-Haemek, Israel) for 4 hours. Transwell plate membranes (Corning Life Sciences, Tewksbury, MA) were coated with fibronectin (Biological Industries, Kibbutz Beit-Haemek, Israel) for 1 hours in 37 degrees. Three hundred and fifty μL of medium with or without the released SDF-1 were added to the bottom chambers of the transwell plates. Three hundred μL of medium containing $1.5x10^5$ cells were added to the top chamber and allowed to migrate for 2.5 hours in 37^0C, 5% CO_2. Medium from the bottom chamber was collected and cell numbers were counted by a hemocytometer (n≥5 in each group).

The Effect of released dexamethasone on NO production in macrophages. RAW 264.7 cells (American Type Culture Collection (ATCC)) were cultured at 37°C in a humidified, 5% carbon dioxide atmosphere in Dulbecco's modified Eagle medium (without phenol red) supplemented with 10% heat-inactivated fetal bovine serum, penicillin (100 units/ml), streptomycin (100 μg/ml) and L-glutamine (2mM) (Biological Industries, Israel). $3.25x10^4$ RAW 264.7 cells in a total volume of 60 μl were seeded per well in 96 well plate. Twenty four hours later, the cells were supplemented with additional 20 μl medium with or without the released dexamethasone. Following 24 hours, 20 μl of growth media containing 25 ng/ml of IFN gamma (PeproTech) were added to each well, reaching a final concentration of 5 ng/ml. After 24 hours incubation, 50μl samples of cell growth medium were analyzed for nitrite concentration (used as an indicator of NO production) using Griess reagent (Promega). Cell viability was determined by Thiazolyl Blue Tetrazolium Bromide (MTT) assay (n≥5 in each group).

Fiber Diameter analysis. Images of fibers were taken using a SEM as described before and fiber diameter analyzed using ImageJ software (NIH).

Cardiac cell isolation, seeding and cultivation. Cardiac cells were isolated according to Tel Aviv University ethical use protocols as previously described [S3]. Briefly, left ventricles of 0–3-day-old neonatal Sprague–Dawley rats (Harlan Laboratories, Israel) were harvested, and cells were isolated using six cycles (30 min each at 37° C) of enzyme digestion with collagenase type II (95 U/mL; Worthington, Lakewood, NJ) and pancreatin (0.6 mg/mL; Sigma–Aldrich) in Dulbecco's modified Eagle Medium (DMEM, $CaCl_2 \cdot 2H_20$ (1.8 mM), KCl (5.36 mM), $MgSO_4 \cdot 7H_2O$ (0.81 mM), NaCl (0.1 M), $NaHCO_3$ (0.44 mM), NaH_2PO_4 (0.9 mM)). After each round of digestion cells were centrifuged (600G, 5 min) and resuspended in culture medium composed of M-199 supplemented with 0.6mM $CuSO_45 \cdot H_2O$, 0.5mM $ZnSO_4 \cdot 7H_2O$, 1.5mM vitamin B12, 500 U/mL Penicillin and 100 mg/mL streptomycin, and 0.5% (v/v) FBS. To enrich the cardiomyocytes population, cells were suspended in culture medium with 5% FBS and pre-plated twice (45 min). Cell number and viability were determined by a hemocytometer and trypan blue exclusion assay. Prior to cell seeding the devices were incubated in a solution of 10 μg/ml of fibronectin (Biological Industries, Kibbutz Beit-Haemek, Israel) in PBS for 24 h in order to improve cell adhesion to the device.

Two million cardiac cells were seeded onto the devices by adding 30 μL of the suspended cells followed by a 1 h incubation period (37^0C, 5% CO_2). Cells were suspended in 50% culture medium and 50% Matrigel (Corning Life Sciences, Tewksbury, MA). Following, cell constructs were supplemented with culture medium (5% FBS) and further incubated.

Immunostaining. Immunostaining was performed as previously described[S3]. Cardiac cell constructs were fixed and permeabilized in 100% cold methanol for 10 min, washed three times in DMEM-based buffer and then blocked for 1 h at room temperature in DMEM-based buffer containing 2% FBS, after which the samples were washed three times. The samples were then

incubated with primary mouse anti α-sarcomeric actinin antibody (1:750, Sigma–Aldrich), washed three times and incubated for 1 h with Alexa Fluor 647 conjugated goat anti-mouse antibody (1:500; Jackson, West Grove, PA). For nuclei detection, the cells were incubated for 3 min with Hoechst 33258 (1:100; Sigma) and washed three times. Samples were visualized using an inverted fluorescence microscope (Nikon Eclipse TI) or a scanning laser confocal microscope (Nikon).

Cell viability. In order to assess cell viability, microECPs were compared to pristine PCL/Gelatin scaffolds ($n \geq 3$ in each group). A PrestoBlue cell viability assay (Life Technologies, NY) was performed. Samples were incubated in a media containing 10% PrestoBlue solution for 6 hours, after which a sample was taken and absorbance at 570 and 600 nm was measured using a Biotek Synergy plate reader (Biotek, Winooski, VT).

Gel degradation assay. Chondroitin sulfate gels with a 1 cm diameter were placed in PBS between two carbon electrodes with a distance of 1 cm between them. Each gel was stimulated for 10 minutes in its respective voltage after which it was freeze dried in a lyophilizer and weighed. This was repeated for 3 cycles.

Cyclic voltammetry. Cyclic voltammograms were recorded with a PalmSens potentiostat/galvanostat (PalmSens, Utrecht, Netherlands) in a 0.1 M potassium nitrate solution containing 5 mM of ferrocyanide/ferricyanide redox couple at a scan rate of 50 mV/s using square PPy-coated microelectrodes, a platinum counter electrode, and an Ag/AgCl quasi-reference electrode.

***In-vivo* implantation and histology.** Recipient SD male rats (150-200 g, Harlan Laboratories, Israel) were anesthetized using a combination of Ketamine (40mg/kg) and Xylazine (10mg/kg). Subcutanuous implantation of samples was performed by creating a small incision to the back.

MicroECPs or pristine PCL scaffolds were folded and inserted into the cavity created by the incision. Three weeks after transplantation the rats were sacrificed and the samples were extracted, fixed in formalin and paraffin embedded. Five μm thick sections were prepared using a microtome and stained with hematoxylin and eosin. Samples were visualized using an inverted fluorescence microscope (Nikon Eclipse TI).